

## Effect of short-term anoxic treatment on internal browning and antioxidant ability in pineapple cv. Phulae

<sup>1</sup>Phonyiam, O. <sup>2</sup>Kongsuwan, A. and <sup>1\*</sup>Setha, S.

<sup>1</sup>School of Agro-Industry, Mae Fah Luang University, Chiang Rai, Thailand

<sup>2</sup>Graduate School of Horticulture, Chiba University, Matsudo, Chiba, Japan

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### Abstract

The effect of short-term anoxic treatment on antioxidant ability and reduction of internal browning in pineapple cv. Phulae was investigated. Fruits previously exposed to pure N<sub>2</sub> gas at flow rate of approximately 700-900 ml/min for 24 hours were packed in polypropylene bags (OTR = 3,060 cc/m<sup>2</sup>·day), placed in corrugated boxes and stored at 10°C for 28 days and subsequently held for 3 days at 25°C. Untreated fruits served as the control. Internal browning and changes in malondialdehyde (MDA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), glutathione, and vitamin C contents, activities of superoxide dismutase (SOD) and catalase (CAT), and antioxidant activities measured by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays were observed during storage. The result showed that 24 hours of anoxic treatment significantly reduced internal browning, while maintaining the quality of Phulae pineapple compared with untreated fruits. MDA and H<sub>2</sub>O<sub>2</sub> contents were lower in anoxic treated fruits compared with untreated while glutathione and vitamin C contents were higher. Changes of SOD activities were not significantly affected by anoxic treatment but CAT activities was initially higher in treated fruit compared with untreated until 14 days of storage. Scavenging activities by DPPH and FRAP assays increased with storage time and they were significantly higher in anoxic treated fruit than non-treated when fruits were held at 25°C. Short-term anoxic treatment reduced internal browning and maintained overall acceptance quality of Phulae pineapple by inducing enzymatic and non-enzymatic antioxidant activities thus delaying increases in H<sub>2</sub>O<sub>2</sub> content and lipid peroxidation.

### Keywords

Anoxia

Antioxidant

Pineapple

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### Introduction

The Phulae pineapple is in a Queen-type variety and is popularly grown in Chiang Rai Province, Thailand. This cultivar has small size (150-1000 g) with cylindrical shape, thick skin, crispy flesh, yellow or greenish-yellow skin color and is known for its excellent quality and sensory characteristics. Although Phulae pineapple has high economic value but it is limited by short storage life. Chilling injury symptoms is developed when fruit is exposed to low temperature at 10°C for about three weeks (Setha *et al.*, 2013). Chilling injury symptom in Phulae pineapple include pitting, failure of green shelled fruit to turn yellow and internal flesh browning (Wongjunta *et al.*, 2009; Setha *et al.*, 2013). Partial control of chilling injury development in Phulae pineapple has been obtained by waxing, polyethylene bagging (Wongjunta *et al.*, 2009) and application of the ethylene inhibitor 1-methylcyclopropene (Setha *et al.*, 2013). Recently, short term anoxic treatment has been used as an inexpensive and non-chemical technology to prevent physiological disorders and delay ripening

in several fruits (Fallik *et al.*, 2003; Pesis, 2005; Pesis *et al.*, 2007). Anoxia pretreatment that reduces surface browning by enhancing enzymatic and non-enzymatic antioxidant activity has been observed in fresh-cut Chinese water chestnut (You *et al.*, 2012). Furthermore, Song *et al.* (2009) found that anoxic treatment delays softening of kiwifruit by reducing the increase in lipid peroxidation and enhancing antioxidant activity. In our preliminary investigation, exposing Phulae pineapple to pure nitrogen gas for 24 hours reduced fresh internal browning incidence, delayed changes in fruit peel color, maintained overall acceptance quality and prolonged the storage life (Tacharoen and Setha, 2012). However, the effect of short-term anoxic treatment on antioxidant ability, which relates to storage life extension of pineapple during low temperature storage, is relatively unclear and thus requires to be elucidated. The objectives of this study were to investigate the effect of short-term anoxic treatment on internal browning incidence, the changes of lipid peroxidation, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), glutathione, vitamin C contents, superoxide dismutase (SOD) and catalase (CAT) activities,

\*Corresponding author.

Email: [sutthiwal.set@mfu.ac.th](mailto:sutthiwal.set@mfu.ac.th)

Tel: 66-53916754, 66-53916737

and scavenging activities by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays.

## Materials and Methods

### *Plant material*

Phulae pineapple (*Ananas comosus*) was harvested at the commercial maturity stage from an orchard in Nang-lae district, Chiang Rai Province, Thailand. Fruit was selected for uniform size, color and absence of any defect or disease.

### *Anoxic treatment*

Thirty five fruits were placed in each 60 L closed plastic chamber and flushed with pure N<sub>2</sub> at flow rate of 700 - 800 ml/min for 24 hours. The O<sub>2</sub> concentration in the chamber was determined to be ≤0.05% (v/v) by using headspace gas analyzer (Mode Check Mate 9900, PBI Dansensor). The fruits were maintained for 24 hours in a humidified N<sub>2</sub> stream (750 ml/min) as the treatment exhibited the most beneficial in maintaining fruit quality during low temperature storage in our preliminary investigation. For the control treatment, fruits were kept for 24 hours in humidified air at the same flow rate with anoxic-treatment. After removal from the chambers, fruits were packed into 0.039 mm thick of polypropylene (PP) with oxygen transmission rate at 3,060 cc/m<sup>2</sup> / day. Thereafter, they were placed into corrugated box and stored for 28 days at 10°C, 85-90% relative humidity and subsequently moved to 25°C (shelf life) for 3 days. Fruit prior to N<sub>2</sub> treatment was used as the 0 day sample. Each treatment had six replications and fruits were sampled every 7 days during storage at 10°C and sampled at day 1 and 3 during storage at 25°C. Flesh was immediately frozen in liquid nitrogen and stored below -30°C until the analysis of antioxidant and oxidative enzymes activities.

### *Internal browning*

Fruits were cut longitudinally in half and the incidence of internal browning was evaluated. Internal browning incidence was scored from 0 to 5 according to the percentage of flesh affected as following; 0, free from IB; 1-5 were ≤10, 10-25, 25-50, 50-75 and ≥ 75% of the flesh discolored, respectively. The average IB incidence was calculated for each lot of fruit.

### *H<sub>2</sub>O<sub>2</sub> content*

H<sub>2</sub>O<sub>2</sub> content was analyzed as described by Velikova *et al.* (2000) with slight modifications. Flesh sample (5 g) were homogenized in 10 ml of 1%

trichloroacetic acid. The homogenate was centrifuged at 20,000 g for 15 min at 4°C. The reaction mixture contained 1 ml of supernatant, 3 ml 50 mM phosphate buffer (pH7) and 0.1 ml 1M potassium iodide. The reaction mixture was then mixed for 1 min and measured at 390 nm with a spectrophotometer (Libra S22, BioChrome, England). The amount of H<sub>2</sub>O<sub>2</sub> was calculated by using a standard curve prepared with known concentration of H<sub>2</sub>O<sub>2</sub>.

### *Lipid peroxidation*

Lipid peroxidation was determined by measuring malondialdehyde (MDA) content. Flesh sample (4 g) was homogenized with 20 ml of 10% trichloroacetic acid and centrifuged at 5000 g for 10 min. Supernatant (1 ml) was mixed with 0.5% thiobarbituric acid dissolved in 10% trichloroacetic acid (3 ml). The reaction mixture solution was then heated for 20 min at 95°C in hot water bath and quickly cooled. Cooled solution was clarified by centrifugation at 10,000 g for 10 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. MDA content was calculated using an extinction coefficient of 155 mM<sup>-1</sup>cm<sup>-1</sup> as described by Song *et al.*, 2009.

### *Superoxide dismutase and catalase activities*

Flesh sample (2 g) was homogenized in 20 ml of 0.1 M phosphate buffer pH 7 containing 3 mM EDTA and 1% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15,000g for 15 min at 4°C and the supernatant was used for SOD and CAT activity assays. SOD activity was determined by the inhibition of nitrobluetetrazolium (NBT) reduction measured at 560 nm as described by Velikova *et al.* (2000). Xanthine oxidase was used to generate the superoxide radicals. One SOD unit was defined as the amount of enzyme that inhibited 50% of NBT. SOD activity was expressed as units per mg protein. The CAT activity was determined as the degradation of H<sub>2</sub>O<sub>2</sub> measured at 240 nm according to method of Beers and Sizer (1952). One CAT unit was defined as the amount of enzyme that degrades 1 μM H<sub>2</sub>O<sub>2</sub> per minute at 25°C. The specific activity of enzymes was expressed as units per mg protein. Soluble protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin as the standard.

### *Glutathione content*

Flesh sample (5 g) was homogenized in sodium phosphate buffer (pH 7) and centrifuged at 15,000 g for 15 min at 4°C. The supernatant was

evaluated for glutathione content at 412 nm with a spectrophotometer as described by Owens and Belcher (1965).

#### Vitamin C content

Vitamin C content was measured in fruit juice by standardization of 2, 6-dichloroindophenol following the titration method (AOAC, 2000). Fruit juice (2 ml) was mixed with 1% oxalic acid (5 ml) and was titrated with 2, 6-dichloroindophenol solution until a light rose pink occurred for 5 sec. The amount of 2, 6-dichloroindophenol solution used in the titration was calculated for vitamin C content.

#### Antioxidant activities

Pineapple tissue (10 g) was homogenized with 20 ml distilled water and then centrifuged for 15 min at 15,000 g at 4°C. DPPH activity was measured according to the method described by Duan *et al.* (2011). The scavenging of DPPH radicals by the samples was expressed as micromole equivalent of trolox 100 g-1FW of pineapple. FRAP activity was assayed by a modified version of the method from Benzie and Strain (1996). The mixture solution was measured at 700 nm. For the standard, the extract was replaced with 0-1000 µM ascorbic acid standard. Iron (III) reducing activity was determined as micromole equivalent of ascorbic acid 100 g<sup>-1</sup> FW of pineapple.

#### Statistical analysis

Statistical analysis used the independent sample T-test. Differences at  $P < 0.05$  were considered statistically significant.

## Results

#### Internal browning

Internal browning in Phulae pineapple fruit increased with storage time in untreated fruit. The internal browning was highly accelerated when fruit was held at 25°C. Only slight internal browning was observed in anoxic treated fruit stored at 10°C and the browning symptom did not significantly increase in fruit that was moved to 25°C (Figures 1 and 2).

#### Lipid peroxidation

MDA contents showed similar changes in both untreated and anoxic treated fruits. MDA content gradually increased for the first 14 days after low temperature storage but MDA contents in anoxic treated showed higher value than in non-treated fruits. Thereafter MDA content greatly dropped in both non-treated and anoxic treated. After transferring fruit to 25°C, MDA content significantly increased

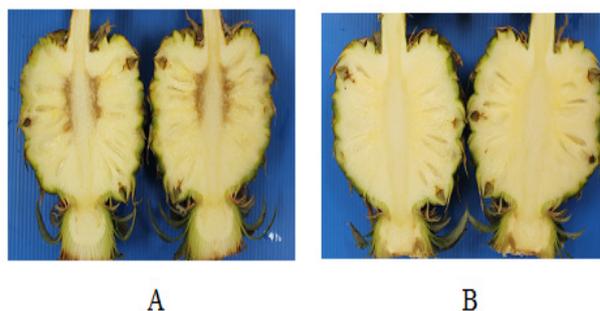


Figure 1. Internal browning appearance in Phulae pineapple stored at 10°C for 28 days (A) untreated and (B) after being held under anoxic conditions for 24 hours

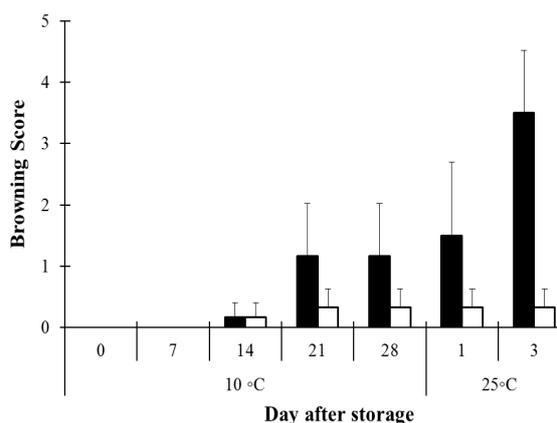


Figure 2. Effect of 24 hour anoxic treatment (□) and untreated (■) on internal browning of 'Phulae' pineapple during storage. Each value is shown as a mean ± standard error (n = 6)

and untreated fruit had significantly higher MDA content than anoxic treated fruit (Figure 3A).

#### H<sub>2</sub>O<sub>2</sub> content

The H<sub>2</sub>O<sub>2</sub> content tended to increase throughout the storage time in untreated fruit, whereas anoxic treatment delayed the increase in H<sub>2</sub>O<sub>2</sub> content of Phulae pineapple. However the H<sub>2</sub>O<sub>2</sub> content in untreated fruit increased after 1 days at 25°C, but its level in anoxic treated fruit remained steady (Figure 3B).

#### Oxidative enzymes

The changes of SOD activity showed similar pattern in both sets of fruit. SOD activity was not affected by anoxic treatment and remained low during storage at 10°C. However, the SOD activity increased sharply after removal to 25°C in both untreated and anoxic treated fruits (Figure 3C). CAT activity highly increased and remained significantly higher in anoxic-treated than untreated fruits during the first 14 days storage at 10°C. Thereafter, CAT activity declined and remained almost the same level as with untreated fruit. The CAT activity slightly

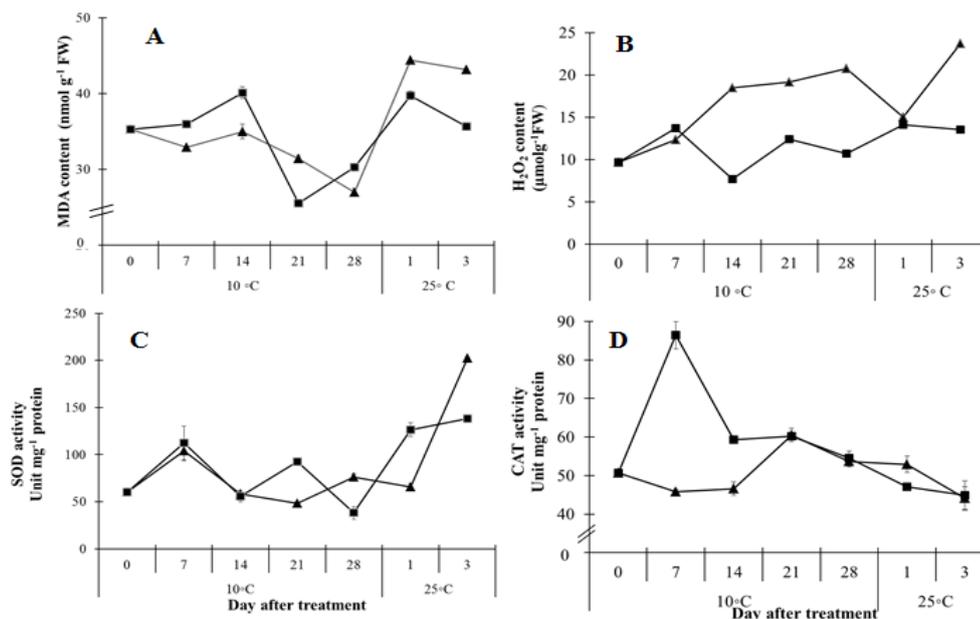


Figure 3. Effect of 24 hour anoxic treatment (■) and untreated (▲) on (A) MDA and (B) H<sub>2</sub>O<sub>2</sub> contents and (C) SOD and (D) CAT activities of 'Phulae' pineapple during storage. Each value is shown as a mean ± standard error (n = 6)

increased from 14 to 21 days in untreated fruit and subsequently declined throughout storage period in all fruits even after transferring to 25°C (Figure 3D).

#### Antioxidant activity

The DPPH scavenging activity highly decreased in all fruits during the first 14 days at 10°C, and then it significantly increased and peaked at day 1 after removal to 25°C and sharply decreased again on the third day of 25°C storage in anoxic treated fruit. The DPPH activity in untreated fruit did not show much change during the time at 25°C (Figure 4A). Overall the anoxic treatment induced higher antioxidant activity by DPPH assay after 28 day of storage than untreated fruit (Figure 4A). FRAP activity increased in the same pattern in both non-treated and anoxic-treated fruit. However, FRAP activity in anoxic treated fruit was generally somewhat higher than in untreated fruit throughout the storage period (Figure 4B).

#### Glutathione and vitamin C contents

Glutathione content in anoxic treated fruit decreased and remained lower than untreated until 21, then sharply increased to 28 days storage at 10°C. It sharply decreased after transferring fruit to 25°C to the same as that in the untreated, then both fruit sets showed similar increases after 3 days of 25°C storage (Figure 4C). Vitamin C content remained fairly steady in anoxic treated fruit but that in untreated decreased throughout the storage period. Vitamin

C content in anoxic treated fruit was significantly higher than untreated from 21 days until the end of storage (Figure 4D).

#### Discussion

Pusittigul *et al.* (2012) found the development of internal browning was higher in Queen-type pineapple cv. Trad-see-thong when fruit was transferred to 25°C than in fruit stored at 10°C. We also found that internal browning increased when fruit was moved to store at 25°C. However, 24 hours anoxic treatment substantially reduced internal browning development in Phulae pineapple. Inhibition of surface browning by anoxic treatment for 4 hours was reported in fresh-cut Chinese water chestnut stored at 4°C (You *et al.*, 2012). The results showed that short term anoxic treatment substantially reduced the internal browning in 'Phulae' pineapple during low temperature storage and subsequent storage at 25°C.

Increase of MDA can reflect the extent of lipid peroxidation induced by oxidative stress or senescence. This study found that the anoxic treatment for 24 hours significantly increased MDA content and this remained higher than in the untreated fruit for the first 14 days during storage at 10°C. However, MDA content in anoxic treated fruit was lower than in untreated when fruit was transferred to 25°C which corresponded to lower internal browning. It suggested that internal browning may not be consistently correlated with changes of MDA

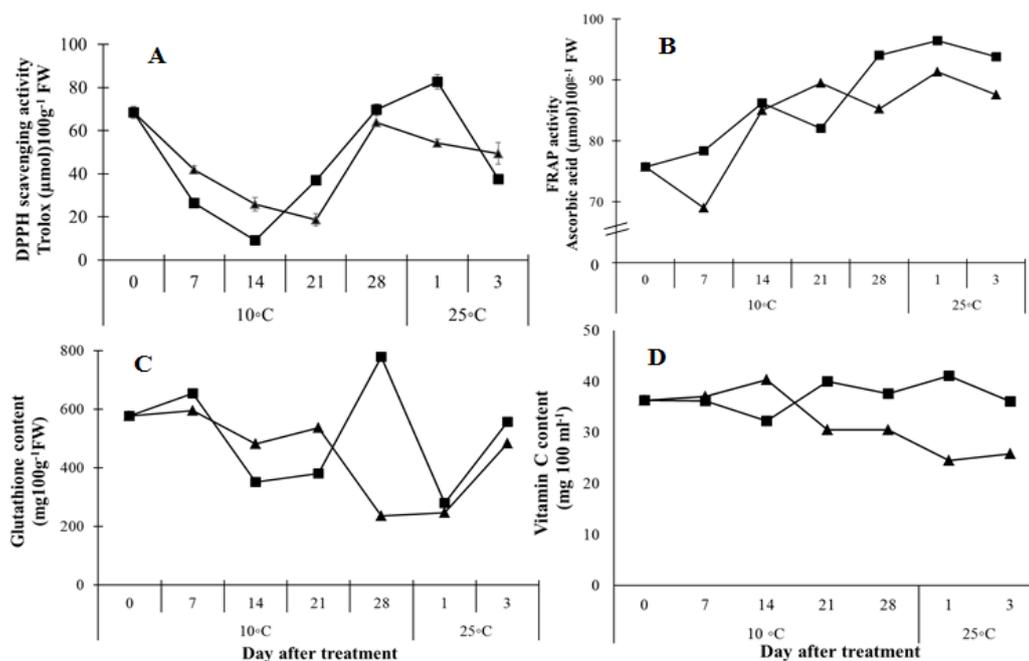


Figure 4. Effect of 24 hour anoxic treatment (■) and untreated (▲) on (A) DPPH and (B) FRAP scavenging activities and (C) glutathione and (D) vitamin C contents of 'Phulae' pineapple during storage. Each value is shown as a mean  $\pm$  standard error ( $n = 6$ )

content during low temperature storage. As similarly reported by Nukuntornprakit *et al.* (2015) that lipid peroxidation, membrane fatty acid composition and the ratio between membrane saturated to unsaturated fatty acid showed no correlation with internal browning in pineapple cv. Trad-see-tong.

The generation of reactive oxygen species (ROS) is considered to be a primary event under a variety of stress conditions (Noctor and Foyer, 1998). Hydrogen peroxide is the first stable compound among ROS produced in the plant cell under normal conditions and as a result of stress (Blokina, 2000). Hydrogen peroxide has been reported to destabilize membranes through substantial lipid peroxidation. According to the idea that  $H_2O_2$  induces chilling injury, in our study we found that  $H_2O_2$  increased and remained at a higher level in untreated fruit than anoxic treated fruit. Increase in  $H_2O_2$  corresponded to the internal browning development. The 24 hours of anoxic treatment can reduce internal browning by reducing or maintaining  $H_2O_2$  production in the cell during low temperature storage.

To protect cells or tissue from the damage by ROS, plant tissues process very efficient enzymatic antioxidant defense systems including SOD and CAT. SOD catalyses the disproportionation of superoxide radical to  $H_2O_2$ , while CAT is involved in eliminating  $H_2O_2$  (Blokina *et al.*, 2003). In this present study, SOD was not affected by anoxic treatment, however the result showed that increase in  $H_2O_2$  levels corresponded to increase of SOD activity.

No correlation was found between chilling symptom and  $H_2O_2$  content, and the activities of SOD or CAT in pineapple cv. Trad-see-thong (Nukuntornprakit *et al.*, 2015). However, CAT activity in anoxic treated Phulae pineapple was induced during the first week of storage and thereafter it declined, which corresponded to increases of  $H_2O_2$  and lipid peroxidation (MDA content). This result suggests that the decrease in CAT activity during storage may be caused by CAT being used up to protect cells from free radical activities. It was also found that short term anoxic treatment increased SOD and peroxidase activities, and reduced lipid peroxidation and thus delayed ripening and senescence of kiwifruit (Song *et al.*, 2009).

In addition, non-enzymatic antioxidants such as ascorbate, glutathione and phenolic compounds play a role in scavenging excessive ROS in plant. Scavenging activity of free radical assessed by DPPH and FRAP assays have been widely used to evaluate the antioxidant activity of natural products (Thaipong *et al.*, 2006; Prabhune *et al.*, 2013). Generally, in this study we found that antioxidant activity in both anoxic treated and untreated fruits increased throughout the storage period. Anoxic treatment showed higher activity than untreated after 2 to 3 weeks storage. The increases in DPPH and FRAP activities in Phulae pineapple corresponded to higher level of internal browning. It is therefore high antioxidant activity should have high efficiency to eliminate free radicals in the plant cell. Nukuntornprakit *et al.* (2015)

concluded that the development of chilling injury symptoms including internal browning correlated with ROS metabolism as reflected in total antioxidant activity. In this study, anoxic treatment increased glutathione content and maintained it at a higher level than in untreated fruit after 3 weeks of storage. Also anoxic treatment maintained higher vitamin C level throughout storage time. Therefore, this suggests that a higher glutathione and ascorbic acid concentration in anoxic treated fruit is beneficial in enhancing non-enzymatic antioxidant activity, thus delaying internal browning in the fruit.

## Conclusion

Short-term anoxic treatment reduced internal browning and maintained overall acceptance quality of Phulae pineapple by inducing enzymatic and non-enzymatic antioxidant activities and this related to delaying increases in H<sub>2</sub>O<sub>2</sub> content and lipid peroxidation.

## Acknowledgements

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